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EFFECT OF COLD HARDENING ON RESISTANCE OF WHEAT SEEDLINGS TO HYDROGEN PEROXIDE AND IRON (II) IONS ACTION.

II. PARTICIPATION OF ANTIOXIDANT ENZYMES

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The activity of antioxidant enzymes in wheat seedlings of Lutescens 329 (cold-resistant) and Bezostaya 1 (cold-sensitive) varieties at their cold hardening (2°C to 6 days) and (or) the action of oxidative stress agents (OS) namely 150 mM hydrogen peroxide or 5 mM iron (II) sulfate was studied. Cold hardening caused an increase in the activity of guaiacol peroxidase in seedlings of both varieties, cold-induced increase in the activity of superoxide dismutase (SOD) and catalase was observed only in seedling of cultivar Lutescens 329. The action of OS agents on the unhardened Lutescens 329 seedlings did not change SOD activity, and guaiacol peroxidase and catalase activity increased. In unhardened Bezostaya 1 seedlings the activity of SOD decreased when processed with the OS agents, catalase activity did not change significantly, and guaiacol peroxidase activity was increased. In hardened seedlings of Lutescens 329 after the action of OS agents there was a significant increase in the activity of SOD and guaiacol peroxidase, while at Bezostaya 1 this effect was almost not manifested. It was concluded the higher cold-induced resistance of Lutescens 329 seedlings to the OS agents and the contribution of enzymatic antioxidants in its manifestation.

Key words: *Triticum aestivum, cold hardening, oxidative stress, hydrogen peroxide, iron, resistance, superoxide dismutase, catalase, guaiacol peroxidase*

Constitutive plant resistance to adverse factors is largely dependent on the functioning of common protective systems, including antioxidant (Shao et al., 2008; Kolupaev, Karpets, 2010; Radyukina et al., 2012). However, the induction of organism resistance by hardening impacts can occur by activation of such non-specific protective systems. In other words, an important component of the induced resistance may be components of non-specific resistance translated to "active" state by the preliminary action of stressor of moderate intensity.

In the previous article we reported on the induction effect of low-temperature hardening on tolerance of cold-resistant wheat Lutescens 329

seedlings to the oxidative stress (OS) agents namely hydrogen peroxide and iron (II) sulfate (Kolupaev et al., 2015b). At the same time the resistance of cold-sensitive Bezostaya 1 seedlings to OS agents after cold hardening changed insignificantly. It was shown that after the action of H₂O₂ and FeSO₄ there was typical a higher sugar and anthocyanin content in hardened seedlings of Lutescens 329 compared with that of hardened ones of Bezostaya 1 (Kolupaev et al., 2015b). However, it can be assumed that enzymatic antioxidants make a certain contribution to the cross-resistance of plants to the action of cold and the OS agents.

Changes in the activity of antioxidant enzymes during cold hardening of plants and cryostress are intensively studied during the last two decades (Scebba et al., 1998). The increased activity of superoxide dismutase (SOD) in wheat seedlings of different varieties under cold hardening

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and the ability of frost-resistant cultivars to maintain the activity of this enzyme in the conditions of cryostress was shown (Ryabchoun et al., 2015). The connection between activity of catalase (Javadian et al., 2010) and different peroxidase (Janda et al., 2003; Major et al., 2010) forms and cold resistance of winter cereals cultivars was detected, although some studies have noted its absence (Apostolova et al., 2008). The possible contribution of enzymatic antioxidant system in the formation of cold-induced plant resistance to other stress factors is still less studied.

The aim of the present study was to investigate the participation of enzymatic components of the antioxidant system in response to treatment with OS agents of unhardened and hardened by cold exposure wheat seedlings of cultivars with different cold resistance.

METHODS

The object of study was etiolated seedlings of winter wheat *Triticum aestivum* L. cultivars *Lutescens* 329 (cold-resistant) and *Bezostaya* 1 (cold-sensitive) obtained from the collection of The National Centre for Plant Genetic Resources of Ukraine (Kharkiv). Technique of plant material preparation to the experiment was described in the previous report (Kolupaev et al., 2015b).

Three-day-old etiolated plantlets were placed for 6 days in the refrigerator compartment Danfoss (Netherlands) for hardening at a temperature of 2°C (Samygin, 1967). As controls, four-day-old seedlings not subjected to hardening were used, since at a low temperature growth of seedlings was slowed and 10-day-old hardened plants corresponded to 4-day ones grown at 20°C.

Control and hardened seedlings were subjected in a two-day exposure to the OS agents – 150 mM hydrogen peroxide or 5 mM iron (II) sulfate (Gaber et al., 2006). After exposure to these substances plantlets were incubated for day (until the end of experiment) on the purified tap water.

Samples were analyzed after hardening, impact of the OS agents and 1 day after its termination. Activity of antioxidant enzymes was determined according to the methods detailed described earlier (Kolupaev et al., 2015a). The sample of plant material (200 mg) was homogenized on the cold in 10 mL of 0,15 M K/Na phosphate buffer (pH 7,6) supplied with 0,1 mM EDTA and 1 mM dithiothreitol. For the analysis, the supernatant prepared by centrifugation of the homogenate at 8000 g for 10 min at 4°C was used. The activity of the cytosolic SOD (EC 1.15.1.1) represented by Cu/Zn-SOD (Alscher et al., 2002) was determined at pH 7,6 of the reaction mixture using

the method based on an ability of the enzyme to compete with tetrazolium nitro blue for the superoxide anions being produced in the course of aerobic interaction of NADH with phenazine metasulphate. The activity of catalase (EC 1.11.1.6) was analyzed at pH 7,0 of the reaction mixture evaluating the amount of the hydrogen peroxide decomposed in a unit of time. The activity of guaiacol peroxidase (EC 1.11.1.7) was determined using guaiacol as a hydrogen donor and hydrogen peroxide as the substrate. The pH of the reaction mixture was brought up to 6,2 with K/Na phosphate buffer.

The protein content in the samples was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

The experiments were repeated independently three times at three biological replicates for each of them. The average values and their standard deviations are shown. Unless otherwise specified the differences significant at $p \leq 0,05$ are discussed.

RESULTS AND DISCUSSION

SOD activity in seedlings of control variant for both cultivars changed insignificantly (table 1). Cold hardening caused increased activity of the enzyme in seedlings of cold-resistant *Lutescens* 329 and slightly affected on it in cold-sensitive *Bezostaya* 1. After transferring the hardened seedlings of both varieties in ordinary temperature conditions SOD activity changed insignificantly.

When the unhardened seedlings of *Lutescens* 329 exposed to OS agents, namely, hydrogen peroxide and iron (II) sulfate, SOD activity did not change significantly, at the same time there was a significant reduction of it in the same seedlings of *Bezostaya* 1 (table 1). Upon termination of the OS agents action on unhardened seedlings the enzyme activity increased slightly in both cultivars.

Preliminary hardened *Lutescens* 329 seedlings answering the action of OS agents (especially hydrogen peroxide) had a significant increase in SOD activity. At the same time, the enzyme activity in hardened seedlings of *Bezostaya* 1 subjected to the action of hydrogen peroxide was higher than that of the corresponding unhardened ones, but lower than that subjected to cold hardening only (without subsequent exposure to OS agents) (table 1). Reaction on iron (II) sulfate treatment of hardened and unhardened *Bezostaya* 1 seedlings did not change significantly. Upon termination of the OS agent action on hardened seedlings of *Bezostaya* 1 the enzyme activity tended to a slight increase, while that of *Lutescens* 329 declined, approaching the control values.

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Table 1. SOD activity (arbitrary units/min/mg protein) in wheat seedlings

Treatment	After 6 days of hardening	After 2 days exposure to OS agents	One day after transfer to water
Lutescens 329			
Control	0,82 ± 0,02	0,92 ± 0,03	0,95 ± 0,04
Hardening	1,01 ± 0,04	0,99 ± 0,04	0,93 ± 0,03
H ₂ O ₂ (150 mM)		0,88 ± 0,02	1,16 ± 0,06
Hardening + H ₂ O ₂ (150 mM)		1,33 ± 0,04	1,05 ± 0,05
FeSO ₄ (5 mM)		0,75 ± 0,05	0,92 ± 0,04
Hardening + FeSO ₄ (5 mM)		1,11 ± 0,03	0,90 ± 0,03
Bezostaya 1			
Control	0,74 ± 0,03	0,77 ± 0,03	0,81 ± 0,02
Hardening	0,85 ± 0,03	0,74 ± 0,04	0,76 ± 0,04
H ₂ O ₂ (150 mM)		0,42 ± 0,02	0,68 ± 0,03
Hardening + H ₂ O ₂ (150 mM)		0,64 ± 0,05	0,72 ± 0,04
FeSO ₄ (5 mM)		0,54 ± 0,03	0,62 ± 0,04
Hardening + FeSO ₄ (5 mM)		0,57 ± 0,04	0,67 ± 0,05

Table 2. Catalase activity (μmol H₂O₂/min/mg protein) in wheat seedlings

Treatment	After 6 days of hardening	After 2 days exposure to OS agents	One day after transfer to water
Lutescens 329			
Control	158 ± 3	167 ± 4	162 ± 3
Hardening	185 ± 2	191 ± 3	159 ± 4
H ₂ O ₂ (150 mM)		204 ± 2	213 ± 6
Hardening + H ₂ O ₂ (150 mM)		220 ± 4	194 ± 4
FeSO ₄ (5 mM)		170 ± 3	166 ± 6
Hardening + FeSO ₄ (5 mM)		181 ± 3	178 ± 4
Bezostaya 1			
Control	161 ± 4	174 ± 6	164 ± 4
Hardening	157 ± 4	187 ± 5	158 ± 4
H ₂ O ₂ (150 mM)		189 ± 4	199 ± 6
Hardening + H ₂ O ₂ (150 mM)		186 ± 4	168 ± 4
FeSO ₄ (5 mM)		183 ± 3	194 ± 6
Hardening + FeSO ₄ (5 mM)		179 ± 5	169 ± 6

Catalase activity in seedlings of control variant for both varieties during the experiment did not change significantly (table 2). Under the influence of cold hardening the enzyme activity increased in seedlings of cold-resistant Lutescens 329 and not changed in those of cold-sensitive Bezostaya 1. In 2 days after hardening the differences in catalase activity between hardened and non-hardened seedlings of variety Lutescens 329 remained, and after 3 days was leveled.

After exposure to hydrogen peroxide (but not iron (II) sulfate) hardened and unhardened seedlings of Lutescens 329 showed the increase in catalase activity. In hardened seedlings, this effect was more pronounced (table 2). After terminating action of hydrogen peroxide the enzyme activity in seedlings of Lutescens 329 remained on elevated level.

In Bezostaya 1 seedlings (as unhardened and hardened) the pronounced changes of catalase ac-

tivity with the action of both OS agents, as well as after its termination, was not observed (table 2).

Guaiacol peroxidase activity in the control variant in seedlings of both cultivars increased during the experiment (table 3) that may be associated with age-related changes in plants (Kolupaev, Karpets, 2006).

Cold hardening caused increased enzyme activity in both studied cultivars (table 3). In 3 days after transferring plants in ordinary temperature conditions guaiacol peroxidase activity of Lutescens 329 variety leveled to corresponding control and that of Bezostaya 1 slightly exceed it.

In unhardened seedlings of both varieties influenced by hydrogen peroxide and iron (II) sulfate the guaiacol peroxidase activity increased (table 3). Hardened seedling of both cultivars had more pronounced reaction on hydrogen peroxide and iron (II) sulfate action, than unhardened ones. Upon terminating OS agents' action in Lutescens 329 seed-

Table 3. Guaiacol peroxidase activity (arbitrary units/min/mg protein) in wheat seedlings

Treatment	After 6 days of hardening	After 2 days exposure to OS agents	One day after transfer to water
Lutescens 329			
Control	57,5 ± 1,9	79,9 ± 2,1	85,5 ± 2,5
Hardening	88,5 ± 2,4	126,0 ± 2,6	85,9 ± 1,8
H ₂ O ₂ (150 mM)		108,5 ± 2,8	102,0 ± 3,8
Hardening + H ₂ O ₂ (150 mM)		153,0 ± 3,4	114,4 ± 2,6
FeSO ₄ (5 mM)		104,0 ± 2,2	89,5 ± 1,0
Hardening + FeSO ₄ (5 mM)		117,5 ± 2,5	99,9 ± 1,4
Bezostaya 1			
Control	49,5 ± 1,9	81,4 ± 4,4	78,2 ± 3,4
Hardening	75,5 ± 3,5	94,7 ± 3,5	94,8 ± 2,4
H ₂ O ₂ (150 mM)		103,0 ± 3,8	112,0 ± 3,0
Hardening + H ₂ O ₂ (150 mM)		118,0 ± 4,2	134,5 ± 3,4
FeSO ₄ (5 mM)		102,5 ± 3,6	113,5 ± 2,5
Hardening + FeSO ₄ (5 mM)		115,5 ± 4,5	135,5 ± 3,0

lings the guaiacol peroxidase activity slightly decreased, approaching the control values. Reaction of Bezostaya 1 seedlings was different: in them after the termination of OS agents' action the guaiacol peroxidase activity increased, especially noticeable in pre-hardened samples (table 3).

Thus, the reaction of the enzymatic antioxidant system components on the action of the OS agents in winter wheat cultivars, differing in cold resistance, was different. Varietal differences manifested significantly after hardening. So, hardened seedlings of Lutescens 329 had a significant increase in activity of SOD, catalase and peroxidase in answer to hydrogen peroxide action (table 1-3). Bezostaya 1 expressed those changes very slightly. The absolute values of the activity of all three enzymes in hardened Bezostaya 1 seedlings in variants with hydrogen peroxide action were markedly lower than those of Lutescens 329.

When using other OS agent – iron (II) sulfate – varietal differences were less noticeable. However, in this case the hardened seedlings of Lutescens 329, unlike those of Bezostaya 1, had a significant increase in SOD activity as well (table 1).

As was stated earlier, cold hardening induces development of resistance of wheat seedlings to the action of OS agents, resulting in less accumulation of lipid peroxidation product malondialdehyde and less inhibiting growth when processing of H₂O₂ or FeSO₄ (Kolupaev *et al.*, 2015b). This effect was well manifested in cold-resistant variety Lutescens 329 and was weakly expressed in cold-sensitive Bezostaya 1.

The obtained results suggest a significant contribution of enzymatic antioxidant system to the cold-induced increase of resistance of wheat seedlings to the action of OS agents. Especially significant differ-

ences under the influence of OS agents between hardened and unhardened seedlings of cold-resistant Lutescens 329 were exhibited in SOD activity (table 1). SOD is the only enzymatic antioxidant, neutralizing superoxide anion radical. It serves the first line of defense against ROS (Alscher *et al.*, 2002). Such SOD function attributed to the fact that, eliminating superoxide radicals, this enzyme indirectly reduces the probability of formation of hydroxyl radicals, singlet oxygen, peroxyinitrite and other ROS, which can not be removed by proteinaceous catalysts by virtue of high reactivity. Several studies have attempted to establish a connection between resistance of different genotypes of plants to the action of stressors and the activity of SOD. Thus, it was shown that cold-resistant rice genotypes responded to the effect of low positive temperatures by increased activity of SOD (Guo *et al.*, 2006). Salt stress activated SOD in leaves of resistant varieties of mulberry and has little effect on the enzyme activity of sensitive genotypes (Xue, Liu, 2008; Ahmad *et al.*, 2010). Higher values of SOD activity observed in seedlings of drought-resistant wheat genotypes, but these varietal differences manifested only after exposure to moderate heat or osmotic stress, inducing development of cross-resistance to hyperthermia and dehydration (Oboznyi *et al.*, 2013). It has been shown that activity of SOD in chloroplasts of higher-yielding and drought-resistant variety of wheat Favoritka was significantly higher than that of less-yielding and non-resistant variety Mironovskaya 808 (Sokolovska-Sergienko *et al.*, 2011). However, such differences were shown only on the background of drought. Thus, the induction of resistance to stressors of various natures is accompanied by increase of SOD activity. Such a reaction is physiologically useful because almost any damaging effects cause amplifica-

tion of the stochastic formation of ROS (Foyer, Noctor, 2009), that can lead to OS development.

The relationship between plant resistance to stressors and the activity of certain enzymes, detoxifying hydrogen peroxide, is apparently not as close. This may be because the H₂O₂, in contrast to superoxide radical, is neutralized by many antioxidant enzymes (Gill, Tuteja, 2010). At the same time, the hardened seedlings of cold-resistant *Lutescens* 329 in our experiments were shown a substantial increase in guaiacol peroxidase activity in response to the OS agents (table 3). In unhardened seedlings of this variety, as well as in hardened and unhardened seedlings of *Bezostaya* 1 this reaction was less pronounced. As already noted, the relationship between the enzyme activity and cold resistance of plants, as well as their resistance to other stressors was detected in a number of papers (Janda et al., 2003; Kapustian et al., 2004; Gill, Tuteja, 2010). We note that the peroxidase is a multifunctional enzyme and its contribution to the plant resistance to stressors can be due not only to ROS detoxification, but also to other reactions, such as those associated with ROS-signaling, post-translational modification of proteins by dimerization of tyrosine residues, changes in the hormonal balance (Maksimov et al., 2011).

Thus, we can say that components of the enzymatic antioxidant system (SOD and possibly other enzymes) are involved in the development of cold hardening-induced resistance of wheat seedlings to the OS agents. Accumulation of low-molecular-weight antioxidants may also be important for the development of induced resistance to the OS. In hardened seedlings of *Lutescens* in response to OS agents the accumulation of anthocyanins was recorded (Kolupaev et al., 2015a). However, hardened seedlings of this variety to the action of the OS agents did not accumulate other low-molecular-weight compounds with antioxidant properties, namely, proline and sugars. At the same time in this paper the hardened seedlings of resistant *Lutescens* 329 showed a marked increase in the activity of antioxidant enzymes. It may be assumed that pre-hardened wheat seedlings of cold-resistant varieties under the influence of OS agents neutralize ROS mainly due to the enzyme component, rather than low-molecular-weight compounds.

Thus, induction by cold of resistance of wheat seedlings to OS includes presence of their ability to activate enzymatic antioxidants. The mechanisms of this phenomenon may be the subject of a special study.

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ВПЛИВ ХОЛОДОВОГО ЗАГАРТУВАННЯ НА СТІЙКІСТЬ ПРОРОСТКІВ ПШЕНИЦІ ДО ДІЇ ПЕРОКСИДУ ВОДНЮ ТА ІОНІВ ЗАЛІЗА (II). II. УЧАСТЬ АНТИОКСИДАНТНИХ ФЕРМЕНТІВ

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Досліджено активність антиоксидантних ферментів в проростках пшениці сортів Лютесценс 329 (морозостійкий) і Безоста 1 (неморозостійкий) за їх холодого загартовування (2°C, 6 діб) та (або) дії агентів окиснювального стресу (ОС) – 150 мМ пероксиду водню або 5 мМ сульфату заліза (II). Холодове загартовування спричинило підвищення активності гваяколпероксидази в проростках обох сортів, індуковане холодом підвищення активності супероксиддисмутази (СОД) і каталази відзначено тільки у проростків сорту Лютесценс 329. За дії агентів ОС на незагартовані проростки сорту Лютесценс 329 активність СОД не змінювалася, а активність каталази і гваяколпероксидази підвищувалася. У незагартованих проростків сорту Безоста 1 за обробки агентами ОС активність СОД знижувалася, активність каталази істотно не змінювалася, а активність гваяколпероксидази підвищувалася. У загартованих проростків

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сорту Лютесценс 329 після дії агентів ОС відзначалося істотне підвищення активності СОД і гваяколпероксидази, в той час як у сорту Безоста 1 такий ефект майже не виявлявся. Зроблено висновок про вищу індуквану холодом стійкість проростків Лютесценс 329 до агентів ОС і внесок ферментативних антиоксидантів в її прояв.

Ключові слова: *Triticum aestivum*, холодове загартовування, окиснювальний стрес, пероксид водню, залізо, стійкість, супероксиддисмутаза, каталаза, гваяколпероксидаза

ВЛИЯНИЕ ХОЛОДОВОГО ЗАКАЛИВАНИЯ НА УСТОЙЧИВОСТЬ ПРОРОСТКОВ ПШЕНИЦЫ К ДЕЙСТВИЮ ПЕРОКСИДА ВОДОРОДА И ИОНОВ ЖЕЛЕЗА (II). II. УЧАСТИЕ АНТИОКСИДАНТНЫХ ФЕРМЕНТОВ

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Исследована активность антиоксидантных ферментов в проростках пшеницы сортов Лютесценс 329 (морозоустойчивый) и Безостая 1 (неморозоустойчивый) при их холодовом закаливании (2°C, 6 суток) и (или) действии агентов окислительного стресса (ОС) – 150 мМ пероксида водорода или 5 мМ сульфата железа (II). Холодовое закаливание вызывало повышение активности гваяколпероксидазы в проростках обоих сортов, индуцированное холодом повышение активности супероксиддисмутазы (СОД) и каталазы отмечено только у проростков сорта Лютесценс 329. При действии агентов ОС на незакаленные проростки сорта Лютесценс 329 активность СОД не изменялась, а активность каталазы и гваяколпероксидазы повышалась. У незакаленных проростков сорта Безостая 1 при обработке агентами ОС активность СОД снижалась, активность каталазы существенно не изменялась, а активность гваяколпероксидазы повышалась. У закаленных проростков сорта Лютесценс 329 после действия агентов ОС отмечалось существенное повышение активности СОД и гваяколпероксидазы, в то время как у сорта Безостая 1 такой эффект почти не проявлялся. Сделано заключение о более высокой индуцированной холодом устойчивости проростков Лютесценс 329 к агентам ОС и вкладе ферментативных антиоксидантов в ее проявление.

Ключевые слова: *Triticum aestivum*, холодовое закаливание, окислительный стресс, пероксид водорода, железо, устойчивость, супероксиддисмутаза, каталаза, гваяколпероксидаза